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Modulation of the tumor microenvironment and inhibition of EGF/EGFR pathway: Novel anti-tumor mechanisms of Cannabidiol in breast cancer

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ABSTRACT

The anti-tumor role and mechanisms of Cannabidiol (CBD), a non-psychotropic cannabinoid compound, are not well studied especially in triple-negative breast cancer (TNBC). In the present study, we analyzed CBD's anti-tumorigenic activity against highly aggressive breast cancer cell lines including TNBC subtype. We show here -for the first time-that CBD significantly inhibits epidermal growth factor (EGF)-induced proliferation and chemotaxis of breast cancer cells. Further studies revealed that CBD inhibits EGF-induced activation of EGFR, ERK, AKT and NF-kB signaling pathways as well as MMP2 and MMP9 secretion. In addition, we demonstrated that CBD inhibits tumor growth and metastasis in different mouse model systems. Analysis of molecular mechanisms revealed that CBD significantly inhibits the recruitment of tumor-associated macrophages in primary tumor stroma and secondary lung metastases. Similarly, our *in vitro* studies showed a significant reduction in the number of migrated RAW 264.7 cells towards the conditioned medium of CBD-treated cancer cells. The conditioned medium of CBD-treated cancer cells also showed lower levels of GM-CSF and CCL3 cytokines which are important for macrophage recruitment and activation. In summary, our study shows -for the first time-that CBD inhibits breast cancer growth and metastasis through novel mechanisms by inhibiting EGF/EGFR signaling and modulating the tumor microenvironment. These results also indicate that CBD can be used as a novel therapeutic option to inhibit growth and metastasis of highly aggressive breast cancer subtypes including

Abbreviations: CBD, Cannabidiol; EGFR, epidermal growth factor receptor; TNBC, triple negative breast cancer; MMP, matrix metalloproteinase; TAMs, tumor associated macrophages.

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TNBC, which currently have limited therapeutic options and are associated with poor prognosis and low survival rates.

1. Introduction

Breast cancer, because of its heterogenous nature, is classified into different subtypes (Perou et al., 2000). TNBC is one of the most aggressive subtypes and is characterized by loss of expression of estrogen, progesterone, and Her2/neu receptors (Bosch et al., 2010). TNBC is known to be unresponsive to estrogen receptor antagonists and Her2 antibody therapies and resistant to its standard chemotherapy (Bosch et al., 2010; Yu et al., 2013).

Cannabinoids can be classified into phyto-cannabinoids that are derived from *Cannabis Sativa*, endogenous cannabinoids that are synthesized inside animal tissues, and synthetic cannabinoids that are produced in laboratories (Shrivastava et al., 2011). CBD is a member of the cannabinoid family and one of the constituents of *Cannabis sativa*. CBD, interestingly, has no psychotropic activity. CBD acts as an inverse agonist for CB2 receptor and an antagonist for CB1 receptor. Additionally, CBD acts as an agonist for TRPV1, TRPV2, TRPA1, PPARγ and 5HT-1_A receptors. Moreover, it works as an antagonist for TRPM8 and GPR55 receptors (Massi et al., 2013). Recently it was reported that CBD down-regulates metastatic factor (ID1) and up-regulates pro-differentiation factor (ID2) (McAllister et al., 2007, 2011). CBD has also been reported to induce programmed cell death in different cancer cells (Ligresti et al., 2006; Marcu et al., 2010; Shrivastava et al., 2011). However, CBD's molecular mechanism of action, its effect on tumor microenvironment and its anti-tumor effect against TNBC are not fully characterized.

TNBC cells are known to highly express basal markers like epidermal growth factor receptor (EGFR) and cytokeratin 5/6 (Gazinska et al., 2013). Higher expression of EGFR in TNBC is associated with poor survival rates (Park et al., 2014). EGFR inhibitors have been used for many solid tumors treatment, inevitably they fail due to development of drug resistance (Wheeler et al., 2010). The tumor microenvironment plays a pivotal role in tumor growth and metastasis (Mantovani and Sica, 2010). Tumor-associated macrophages (TAMs) are well known to contribute principally in tumor progression (Mantovani and Sica, 2010). There are two different forms of macrophages (M1 and M2); M1 are anti-tumorigenic macrophages while M2 have pro-tumorigenic ability (Dijkgraaf et al., 2013).

In the present study, we document inhibitory properties of CBD on growth and metastatic properties of breast cancer cell lines including TNBC *in vitro* and *in vivo*. Furthermore, we show that CBD inhibits EGF-induced proliferation, migration/invasion, and activation of ERK and AKT signaling pathways. We also show that CBD inhibits breast cancer growth and metastasis through modulation of the tumor microenvironment. This study provides insight into novel CBD-mediated anti-tumorigenic/metastatic mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY). The following reagents and antibodies used in this study were purchased from different sources: Cannabidiol (Sigma Aldrich); human/murine EGF (Peprotech); GAPDH, AKT, p-EGFR/EGFR, Arginase-I, CD31, and p-ERK/ERK (Santa Cruz); and F4/80 (Abd Serotec) and Ki67 from (NeoMarkers); and p-AKT from (Cell Signaling). For flow-cytometery studies all antibodies were purchased from (Biolegend). For lung fixation Bouin's reagent was purchased from (Sigma Aldrich).

2.2. Cell culture

Human TNBC cell line SUM159 (Grigoriadis et al., 2012) was kindly provided by (Dr. Sarmila Majumder, The Ohio State University) and 4T1.2, a subclone of murine TNBC cell line 4T1 cells (Jin et al., 2014a; Lelekakis et al., 1999) was kindly provided Dr Robin Anderson (Eckhardt et al., 2005). SCP2, a subclone of MDA-MB-231 cells, was kindly provided by Dr. Joan Massagué (Minn et al., 2005). MVT-1 cell line was obtained from Dr. Johnson (Pei et al., 2004). MDA-MB-231 and RAW 264.7 cell lines were purchased from American Type Culture Collection (ATCC). The identity of these cells was verified regularly on the basis of cell morphology. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 5 units/mL penicillin, and 5 mg/mL streptomycin (Corning Cellgro) and grown in 5% CO_2 incubator at 37 °C.

2.3. Cell proliferation assay

Cells were seeded in 96 well plates and treated with different concentrations of CBD with or without EGF (100 ng/ml) for 48 h in SFM and subjected to MTT assay (Roche) according to manufacturer's protocol.

2.4. Chemotactic assays

Chemotactic assays were performed using transwell chambers (Corning-Costar). Briefly, cells were pretreated with CBD or vehicle. Top chambers were loaded with (1.5×10^5 cells for migration assay) or (2×10^5 cells for invasion assay) in serum-free medium (SFM) and bottom chambers contained SFM in presence or absence of (EGF) or cancer cells conditioned media. Cells that migrated were stained and counted (Wani et al., 2014).

2.5. Immunofluorescence

Immunofluorescence was performed as described earlier (Ravi et al., 2014).

2.6. Flow-cytometry

Single cell suspensions were prepared from tumors (Nasser et al., 2012). Cells were incubated with anti-CD11b APC, anti-F4/80 PE, and anti-CD206 (Alexa Flour-488) for 1 h then fixed with 2% paraformaldehyde and acquired on a BD FACS caliber, then analyzed using Flowjo software.

2.7. Gelatin zymography

Gelatin zymography for collected conditioned media was performed as described earlier (Ravi et al., 2014).

2.8. Luciferase reporter assay

We used NF-kB luciferase reporter assay (Promega) to determine NF-kB activity as described previously (Ravi et al., 2014). Briefly, NF-kB luciferase constructs containing either wild type or NF-kB vector were transfected in the pretreated cells using lipofectamine 2000 (Invitrogen). For internal control, we co-transfected cells with Renilla luciferase vector. 24 h after transfection, EGF (100 ng/ml) was added and then incubated for another 24 h. Cells were lysed and luciferase assay was performed according to manufacturer's protocol.

2.9. Colony forming assay

One thousand cells were seeded in 60 mm^2 plates in DMEM supplied with 10% FBS. The next day the media were changed to DMEM with 3% FBS and incubated for 6 days with or without (EGF 100 ng/ml). Cells were fixed, stained and clones were counted (Ravi et al., 2014).

2.10. Mouse models

Female Balb/C and FVB mice were purchased from (Charles River Laboratories Inc.). Tumors were formed by orthotopically injecting 4T1.2 (1×10^5) or MVT-1 (5×10^5) cells into the 4th mammary glands. When tumors became palpable, mice were randomized and injected peri-tumorally with CBD (10 mg/kg) or vehicle on alternate days for 3 weeks. Tumors were measured every week with external calipers and tumor volume was calculated according to the formula $tumor\ volume = (length \times width^2 \times 0.52)$ (Nasser et al., 2012, 2011).

2.11. Western blot (WB), real time PCR (RT-PCR) and immunohistochemistry (IHC)

WB, RT-PCR and IHC were performed as described earlier (Nasser et al., 2012; Qamri et al., 2009).

2.12. Wound healing assay

Wound healing experiment has been performed as described earlier (Preet et al., 2011). Briefly, Cells were treated with CBD or vehicle for 24 h. Monolayers were scratched with a sterile 200 μL micropipette tip, washed, and incubated in media supplemented with 0.1% FBS in the presence or absence of CBD or vehicle and EGF (100 ng/ml). After another 24 h, cells were fixed and photographed.

2.13. Statistical analysis

Results were represented as mean ± SD. Student's t test was used to compare vehicle and CBD-treated groups. $P < 0.05$ was considered to be statistically significant. For all graphs, * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

3. Results

3.1. CBD inhibits breast cancer cell proliferation, migration and invasion

Cell proliferation is an important characteristic of cancer cells to survive and form a tumor mass (Ravi et al., 2014). As shown, TNBC cells express EGFR (Figure S4), Furthermore, EGF/EGFR axis plays an important role in proliferation of breast cancer cells (Wheeler et al., 2010); therefore, we performed MTT assay to evaluate CBD's effect on cancer cell viability. We found that CBD induces a strong dose-dependent decrease in proliferation of SUM159 and SCP2 human TNBC as well as 4T1.2 murine cell lines after 48 h incubation (Figure 1 (A–C)). We chose lower concentrations (CBD, 3, 6 and 9 μM) that have lower direct effect on proliferation for further studies. We tested the anti-proliferative ability of low doses of CBD in presence of EGF. As shown in Figure 2-A and B, CBD significantly inhibits EGF-induced proliferation in SUM159 and 4T1.2 cells respectively. In order to test the ability of CBD to inhibit cancer cell survival and proliferation to form single cell clones, we performed colony forming assay. We found that CBD significantly inhibited the number of cancer colony forming cells (SUM159 and 4T1.2) respectively after EGF stimulation (Figure 2 C–D).

Migration and invasion are two important characteristics of cancer cells to metastasize and form secondary tumors (Yamaguchi et al., 2005). When there is a gradient of extracellular stimuli like EGF, cells will undergo cytoskeletal rearrangement associated with an increase in cell adhesion, resulting in directional migration (Xie et al., 1998). Therefore, we evaluated the ability of CBD to inhibit EGF-induced migration of 4T1.2 and SUM159. CBD (6 μM) significantly inhibited EGF-induced migration in these cell lines (Figure 2 E–F). To confirm, we analyzed CBD effect in wound healing assay. As expected, CBD 6 μM inhibits EGF-induced wound closure in MDA-MB231 and SUM159 cell lines (Supp. Figure 3 A–B).

The extracellular matrix (ECM) and the basement membrane are barriers of cancer cells that hinder migration and invasion, so we were interested to examine CBD's modulatory effect on cancer cell invasion through ECM. We found that CBD (6 μM) inhibited EGF-induced invasion of SUM159 cells and 4T1.2 cells through ECM and basal membrane coated filters (Figure 2 G–H).

These results suggest that CBD -even at low concentrations- has the ability to inhibit the proliferative, migratory and invasive ability of TNBC cells induced by EGF.

3.2. CBD modulates EGF/EGFR signaling

EGFR signaling pathways are known to activate many cellular targets that are important for cancer cell survival, migration,

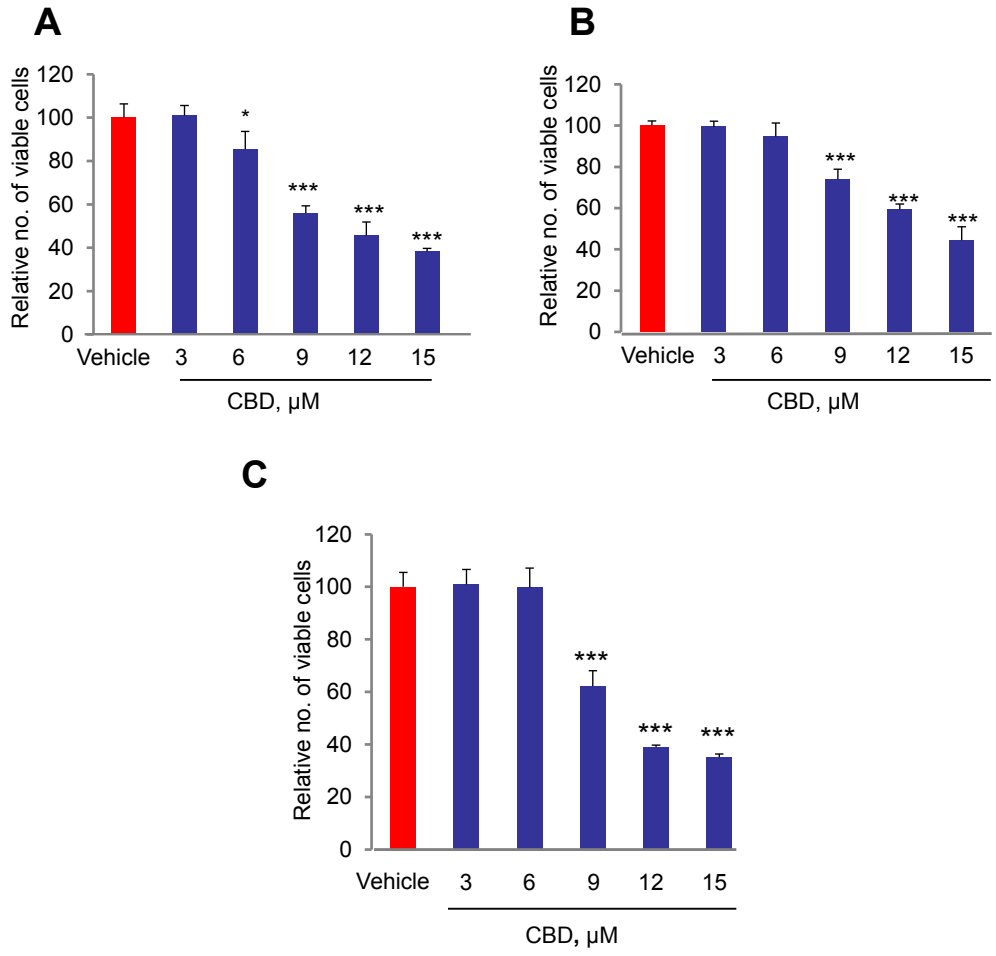

Figure 1 – CBD inhibits proliferation of breast cancer cells. SUM159 (A), 4T1.2 (B) and SCP2 (C) cells were treated with CBD (3–15) μM for 48 h and then subjected to MTT assay. Measurements were plotted as % of control.

and invasion. Both AKT and ERK are downstream targets of the EGF/EGFR pathway and they are crucial for cell growth and metastasis (Seshacharyulu et al., 2012). NF-kB activation is well known to promote the migration and invasion of TNBC cells (Helbig et al., 2003; Singel et al., 2014). Furthermore, there is a correlation between high levels of NF-kB activation and EGFR overexpression in breast cancer (Biswas and Iglehart, 2006). Therefore, we examined the ability of CBD to inhibit NF-kB signaling. As shown in Figure 3-A, CBD 6 μM inhibited EGF-induced translocation of NF-kB into the nucleus in SUM159 cells compared to vehicle-treated cells. To further understand the molecular mechanism by which CBD modulates the EGF/EGFR pathway, we treated SUM159 and 4T1.2 with CBD 6 μM for 48 h and then we stimulated the cells with EGF (100 ng/ml) for 15 and 30 min. As shown, CBD (6 μM) inhibited EGF-induced phosphorylation of EGFR, AKT and ERK in SUM159 (Figure 3-B). We also showed inhibition of phosphorylation of AKT and ERK by CBD after EGF stimulation of 4T1.2 cells (Supp. Figure 1-A).

EGF has also been reported to induce tumor cell invasion through induction of matrix metalloproteinases (Kim et al., 2009; Xu et al., 2011). In order to understand whether CBD-mediated inhibition of invasion is due to CBD effect on MMPs, we treated cells with CBD and analyzed MMPs activity in cancer cell-conditioned media by Zymography. As shown, CBD has reduced MMP2 and MMP9 activities in SUM159 and 4T1.2 respectively (Supp. Figure 1 B–C).

Cancer cells make protrusions to help them in adhesion and migration, and these protrusions are rich in actin filaments and adhesion molecules (Yamaguchi and Condeelis, 2007). We examined the effect of CBD on EGF-induced actin stress fiber and focal adhesion formation. By immunofluorescence, we showed that CBD 6 μM inhibits EGF-induced actin stress fiber formation as shown by changes of Phalloidin expression and focal adhesion formation as detected by changes in Vinculin expression in SUM159 cells (Figure 3-C).

These results indicate that CBD can inhibit EGF-induced tumorigenic properties of cancer cells through inhibition of the activation of EGFR, AKT, ERK and NF-kB signaling; in addition it blocks MMPs secretion, and suppresses phalloidin expression and actin stress fiber formation induced by EGF.

3.3. *CBD inhibits tumor growth in breast cancer mouse models*

To confirm the *in vitro* data, we evaluated CBD ability to inhibit breast cancer growth *in vivo*. We found that CBD inhibited tumor growth, in 4T1.2 mouse model, as shown by reduction in

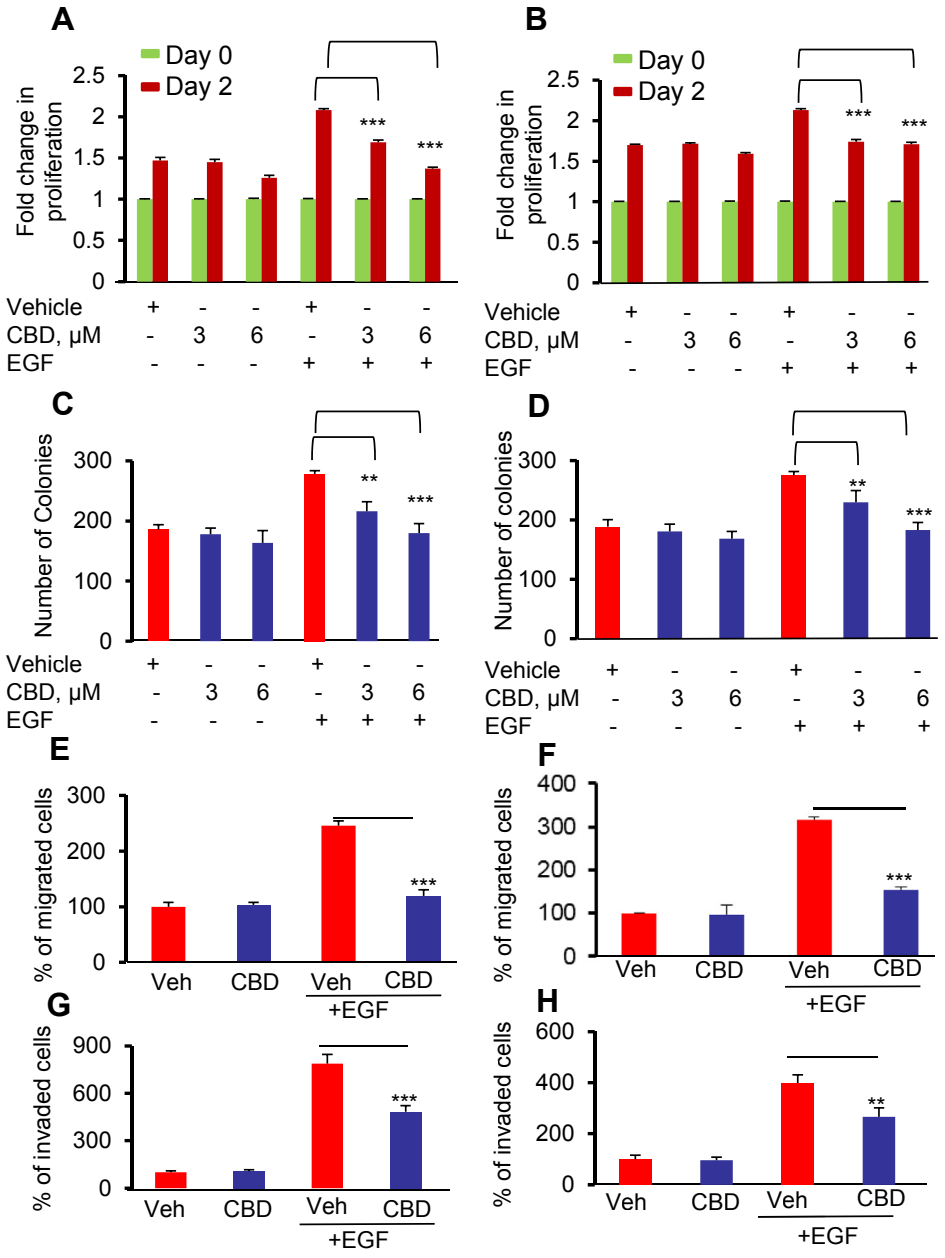

Figure 2 – CBD inhibits EGF induced breast cancer EGF-induced cell proliferation, colony formation, migration and invasion. . SUM159 (A) or 4T1.2 (B) cells were treated with vehicle or CBD (3 and 6) μM with or without EGF (100 ng/ml) for 48 h and subjected to MTT assay. Data represent fold change of proliferation after 48 h (Day 2) relative to basal level of proliferation (Day 0). Colony forming assay was performed for SUM159 (C) or 4T1.2 (D) cells that were treated with vehicle or CBD with or without EGF (100 ng/ml). SUM159 (E) or 4T1.2 (F) cells were treated with CBD 6 μM for 48 h and subjected to transwell migration assay. SUM159 (G) or 4T1.2 (H) cells were treated with CBD 6 μM for 48 h and subjected to transwell invasion assay. Number of migrated or invaded cells were counted and plotted as % of control.

tumor volume (Figure 4-A) and weight (Figure 4-C) compared to control group. By IHC staining, we observed a dramatic decrease in the tumor cell proliferation, tumor vascularization and p-EGFR expression in CBD-treated group (Figure 4-G). To further investigate CBD's mechanism of tumor suppression, we used the tumor lysates to examine CBD effect on signaling pathways *in vivo*. CBD treated tumors showed decreased phosphorylation of AKT and ERK proteins (Figure 4-I).

We confirmed *in vivo* results using another highly aggressive mouse cell line (MVT-1). CBD treatment (10 mg/kg) significantly reduced tumor volume and weight in MVT1-1 model (Figure 4-B and D, respectively). We also analyzed Ki67, CD31 and p-EGFR levels by IHC staining. CBD-treated tumors had decreased proliferative activity, vessel formation and p-EGFR expression (Figure 4-H). Furthermore, CBD-treated group also showed less activation of AKT, and ERK proteins in MVT-1 tumor lysates compared to the control group (Figure 4-J).

These results suggest that CBD has the potential to inhibit tumor growth through suppression of tumor cell proliferation, angiogenic potential and inhibition of the activation of EGFR, AKT and ERK proteins.

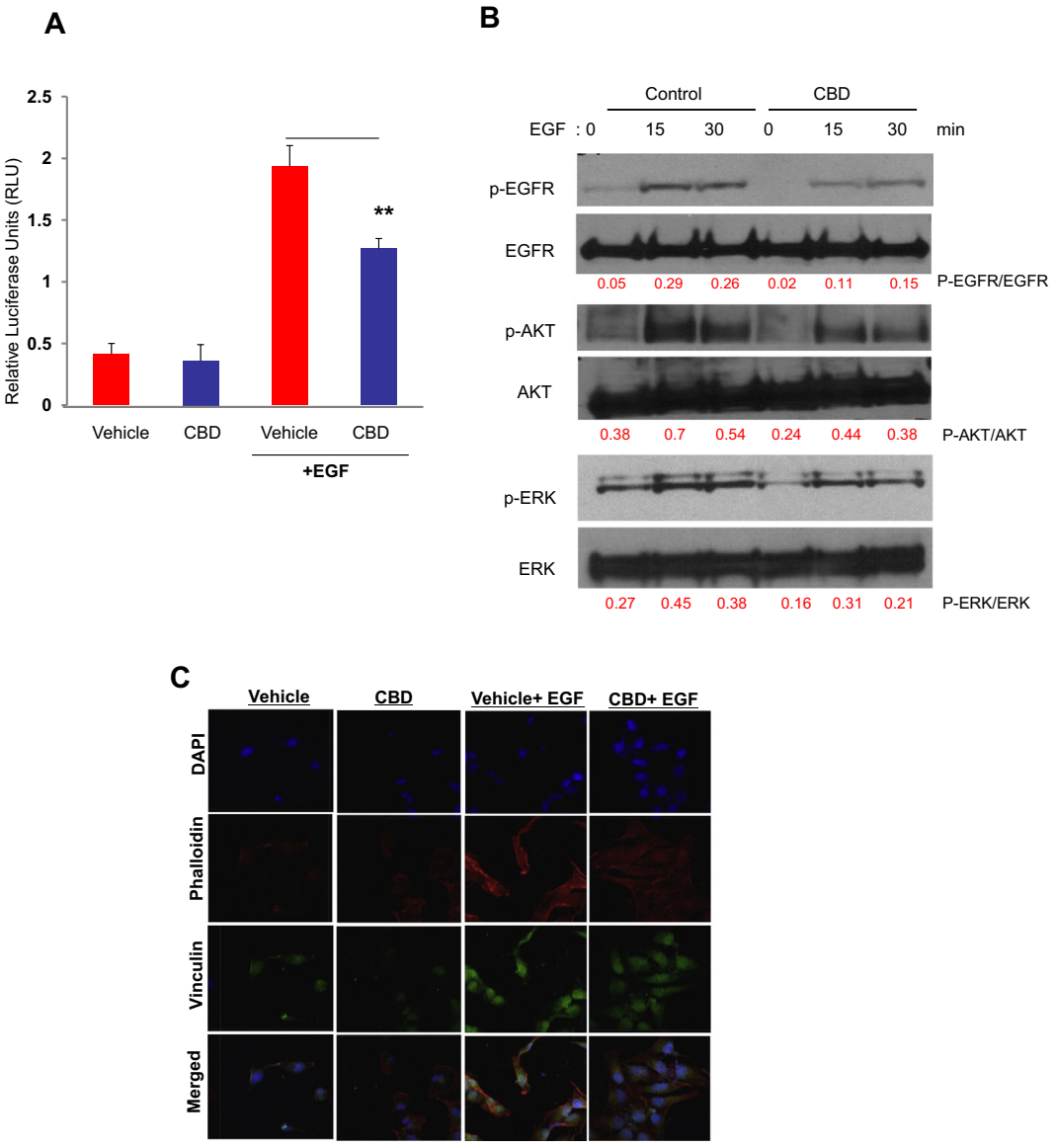

Figure 3 – CBD inhibits EGF/EGFR signaling. (A) SUM159 cells were treated with vehicle or CBD 6 μM in the presence or absence of EGF and subjected to NF-kB luciferase reporter assay. (B) SUM159 cells were treated with CBD 6 μM, stimulated with EGF for 15 or 30 min then cell lysates were used for western blot analysis for the indicated proteins. Relative expression of p-EGFR/EGFR, p-AKT/AKT and p-ERK/ERK has been quantified by image-j software and provided as numbers (indicated in red color) (C) Confocal microscopy visualization of SUM159 cells treated with vehicle or CBD 6 μM and stimulated with EGF (100 ng/ml) and stained for phalloidin (red), vinculin (green) and DAPI (blue).

3.4. *CBD inhibits metastasis of breast cancer cells to the lung*

Breast cancer patients' survival is limited in part by the development of distant metastases (Jin et al., 2014b). To study the efficacy of CBD to inhibit metastasis, two highly aggressive breast cancer cell lines (4T1.2 and MVT-1) were employed. The 4T1.2 subclone is known for its propensity to metastasize with rates superior to that of its parental cell line (4T1) (Lelekakis et al., 1999). After 3 weeks of treatment, CBD-treated groups showed significantly less number of metastatic nodules and less total lung weight in 4T1.2 and MVT-1 mouse models than control groups (Figure 5 A–F) and (Supp. Figure 5 A–D).

Since tumor cells secrete MMPs which enhance their ability to invade and metastasize to distant organs (Stamenkovic, 2000), we analyzed MMP2 and MMP9 expressions in tumor lysates of CBD-treated and control groups. The CBD-treated group had significantly lower expression of MMP2 and MMP9 in both 4T1.2 and MVT-1 tumors (Figure 5 G–H), suggesting that the anti-metastatic effects of CBD are likely mediated through decreased MMP2 and MMP9 secretion by the tumor cells.

3.5. *CBD inhibits tumor growth and metastasis through inhibition of macrophage recruitment to tumor sites*

Since tumor-associated macrophages (TAMs) are known to modulate tumor angiogenesis, cancer cell proliferation, and invasion (Place et al., 2011), we investigated CBD's effect on macrophage populations within the breast tumor microenvironment. First, we examined breast cancer xenografts for

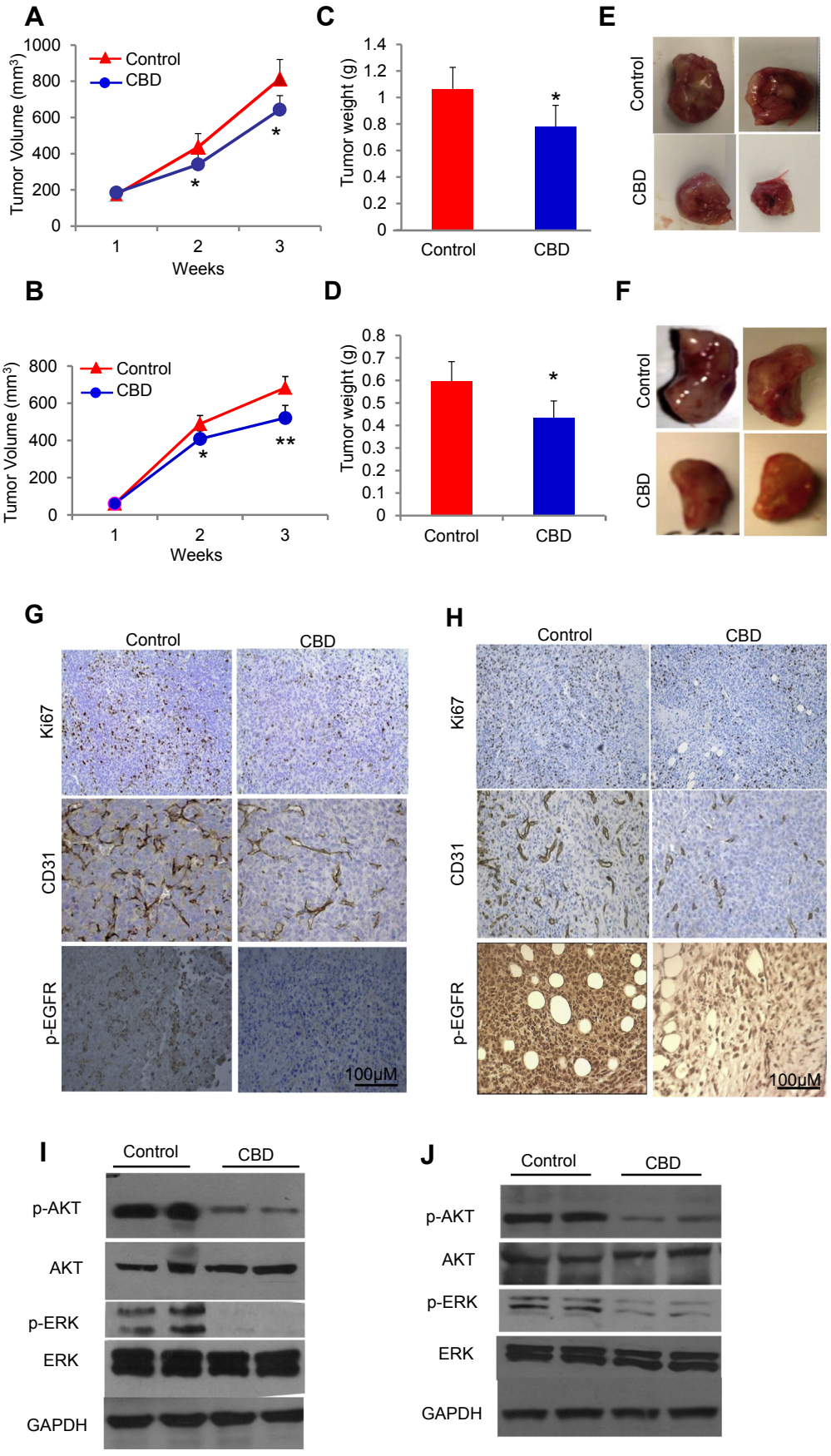

Figure 4 – CBD inhibits breast tumor growth in different mouse model systems. Tumor volume measurements of 4T1.2 (A) or MVT-1 (B) mouse models were assessed every week for control and treated groups. Tumor weight of various experimental groups was determined in 4T1.2 (C) or

total (F4/80-positive) and M2 (Arginase-I-positive) macrophages by IHC. CBD-treated tumors showed decreased F4/80 and Arginase-I-positive cells compared to control group (Figure 6-A and B). Next, we analyzed macrophage populations in the primary tumors by flow-cytometry. The CBD-treated tumors have significantly smaller percentages of total macrophage ($CD11b^+$, $F4/80^+$) in MVT-1 and 4T1.2 tumors (Figure 6-C and Supp. Figure 2, respectively). Decreased percentage of M2 ($F4/80^+$, $CD206^+$) macrophages are detected in CBD-treated tumors of MVT-1 tumor-bearing mice (Figure 6-D).

Macrophages facilitate tumor angiogenesis, ECM breakdown, and cancer cell motility, which are crucial components of the metastatic process (Pollard, 2004). Furthermore, macrophages are needed for metastatic cancer cell survival and formation of pre-metastatic niche (Gil-Bernabé et al., 2012). Therefore, to investigate CBD effect on macrophage populations within secondary lung metastases, we analyzed F4/80 and arginase-I expressions in the lungs of vehicle- and CBD-treated groups by IHC staining. As expected, CBD-treated group had less F4/80 and Arginase-I expression in the lung metastases compared to vehicle-treated groups (Figure 6 E–F).

In order to understand why there is a reduction of macrophage population within the tumor, we treated 4T1.2 cells with CBD 9 μM or vehicle for 48 h, and the collected conditioned media were used as a chemo-attractant to test migration of RAW 264.7 cells. We observed a significant reduction in the number of migrated RAW 264.7 cells towards CBD-conditioned medium compared to the control-conditioned medium (Figure 7-C). We hypothesized that CBD might modulate cytokine production from tumor cells, which in turn affects macrophage recruitment towards the cancer cells. To test this hypothesis, we performed cytokine array analysis (R&D Systems), using the previously mentioned conditioned media. Interestingly, we found that CBD-treated 4T1.2 cells secreted less CCL3, GM-CSF and MIP-2 proteins compared to vehicle-treated cells (Figure 7 A–B).

These results suggest that CBD modulates cytokine production from tumor cells which leads to less recruitment of total macrophages and M2 macrophages into the primary and secondary tumor sites. This partially explains the ability of CBD to modulate the breast tumor microenvironment which helps in inhibition of tumor progression and metastasis to distant organs.

4. Discussion

Aggressive breast cancer subtypes are characterized by high proliferative and metastatic index and very poor prognosis. TNBC is one of the highly aggressive breast cancer subtypes that constitutes 12–24% of all breast cancer subtypes and shows poor relapse-free and overall survival, as reviewed previously (Bosch et al., 2010). In contrast to other breast cancer subtypes, TNBC lacks molecular targets amendable to specific therapeutic agent (Cleator et al., 2007; Nogi et al., 2009). Chemotherapy still is the first line of treatment for TNBC, however rapid development of resistance and the poor response rate are common (Yu et al., 2013), thus highlighting an urgent need for novel therapeutic therapies.

Cannabinoids have been used in cancer therapy owing to their ability to modulate pain, as well as their anti-inflammatory, cell growth inhibition, and apoptotic effects (Pisanti et al., 2009). CBD is a phytocannabinoid that constitutes about 40% of *cannabis sativa* extract. Although CBD exerts strong anti-tumorigenic activity in various cancer types, CBD's anti-tumor effect on highly aggressive and metastatic breast cancer cells including TNBC subtype and its influence on the EGF/EGFR pathway and the tumor microenvironment still needs to be clarified. In the present study, we investigated the molecular basis of CBD's anti-tumor activity. We showed that CBD inhibits breast cancer growth and metastasis, especially the TNBC subtype. To our knowledge, we discovered for the first time that CBD has the ability to inhibit EGF-induced tumorigenesis. We also showed – for the first time – that CBD has the ability to inhibit breast cancer growth and metastasis through modulation of the tumor microenvironment.

The EGF/EGFR axis plays an important role in cancer cell survival, growth, metastasis and angiogenesis (Capdevila et al., 2009; Zhang et al., 2007). EGFR is known to be overexpressed in TNBC. Current EGFR inhibitors have been shown to get rapid resistance in various cancer cell types (Wheeler et al., 2010). Therefore, it is important to identify novel drugs that can target this axis in order to inhibit cancer growth and metastasis mediated by the EGF/EGFR pathway, especially in TNBC patients. In our present study, we showed that CBD suppressed the activation of EGF/EGFR signaling transduction pathways in highly aggressive and metastatic breast cancer cells including TNBC subtype *in vitro*. These pathways involve two key molecules (ERK and AKT) which are known to be important survival molecules. This explains the ability of CBD to inhibit EGF-induced proliferation, migration, and invasion. This finding is especially important because it is well known that EGFR overexpression is associated with poor prognosis, especially with TNBC patients (Park et al., 2014).

Aberrant activation of EGF/EGFR signaling activates the NF-kB pathway, which in turn has a tumor pro-survival effect with resistance to chemotherapy (Tanaka et al., 2011). Here, we reported that CBD inhibits EGF-induced activation of NF-kB. This effect might be highly useful, as it is clear that inhibition of EGF-induced activation of NF-kB should be an important anti-tumor target and it explains as well the ability of CBD to inhibit EGF-induced breast cancer cell proliferation. It is also known that NF-kB has an important role in breast cancer metastasis through multitarget effects. It was reported that NF-kB can induce CXCR-4 expression which is a well-known receptor needed for breast cancer cell migration through binding to its ligand (SDF-1alpha) (Helbig et al., 2003). It was also shown that NF-kB activation is required for epithelial–mesenchymal transition (EMT) process and inhibition of

MVT-1 (D) mouse models. Representative photographs showing tumors dissected from various experimental groups of 4T1.2 (E) or MVT-1 (F) mouse models. Representative photomicrographs of immunostaining with Ki67, CD31 and phospho-EGFR (p-EGFR) of tumors of control and CBD-treated groups in 4T1.2 (G) or MVT-1 (H) mouse models. Western blot images of 4T1.2 (I) or MVT-1 (J) tumors showing the expression of phospho-ERK or AKT (p-ERK, p-AKT) and total ERK and AKT (ERK, AKT) proteins in control and CBD-treated groups.

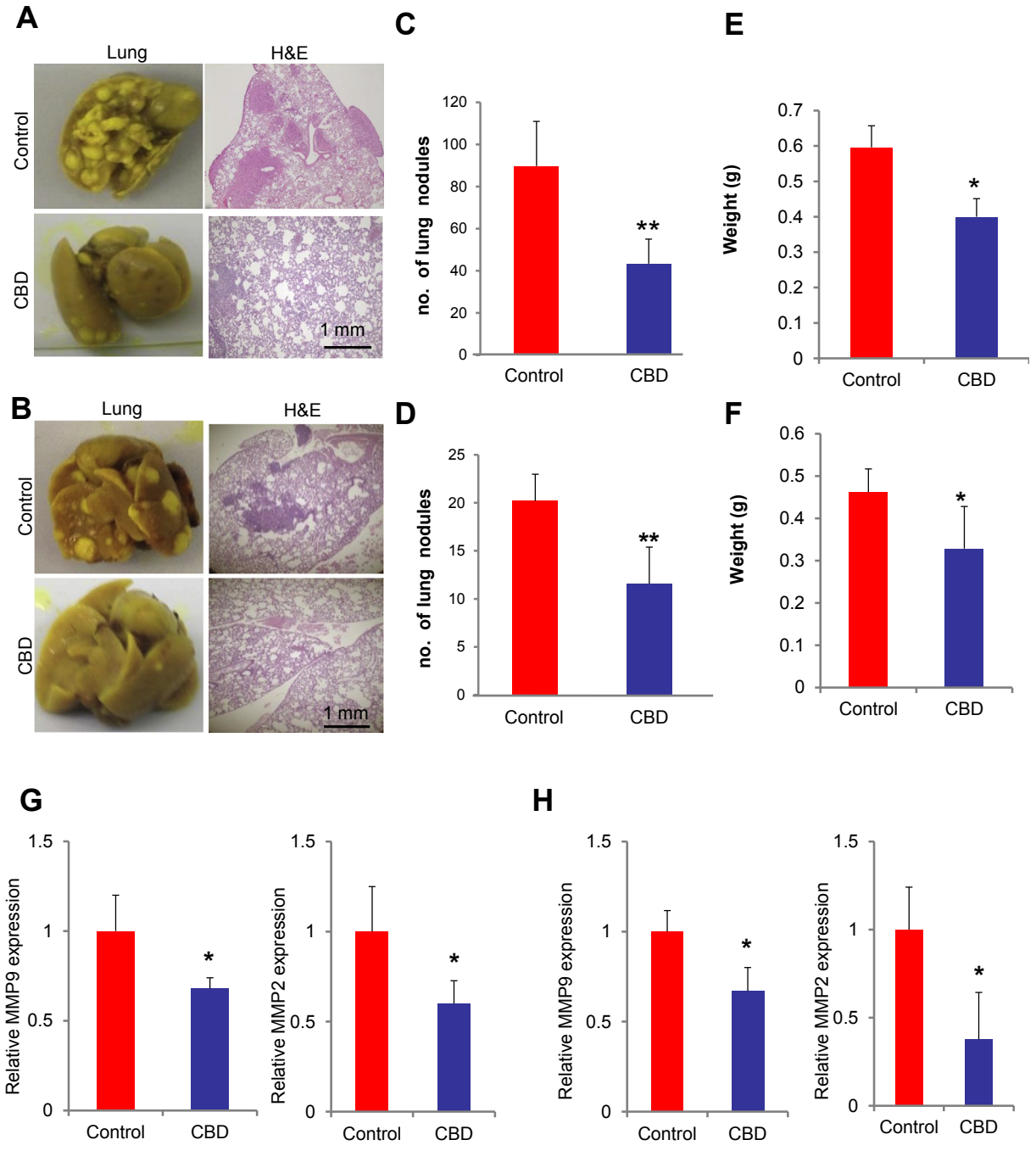

Figure 5 – CBD inhibits lung metastasis in different mouse model systems. Representative lung images and H&E staining photomicrographs were taken of control and CBD-treated groups of 4T1.2 (A) and MVT-1 (B) mouse models. The number of metastatic lung nodules was counted for 4T1.2 (C) and MVT-1 (D) mouse models of control and CBD-treated groups. Total lung weight was determined for 4T1.2 (E) and MVT-1 (F) mouse models of control and CBD-treated groups. RT-PCR quantification was performed for MMP-9 and MMP-2 in 4T1.2 (G) and MVT-1 (H) mouse models in control and CBD-treated tumors 18S rRNA primers were used for loading control purpose.

NF-kB signaling prevented EMT and significantly reduced metastatic potential of breast cancer cells (Huber et al., 2004). Interestingly, NF-KB activation has been shown to promote bone metastasis through GM-CSF induction (Park et al., 2006) which it might explain the lower level of secreted GM-CSF from CBD-treated cancer cells and suggests that CBD may play a role in inhibition of breast cancer metastasis to bone.

Adhesion molecules are known to connect actin stress fibers to ECM, and therefore they help in regulation of cancer cell migration (Yamaguchi et al., 2005). Reorganization of the actin cytoskeleton can lead to stress fiber assembly which in turn modulates the migration and invasion of cancer cells (Friedl and Wolf, 2003). Metalloproteinases are well-known to hydrolyze ECM and basement membrane components

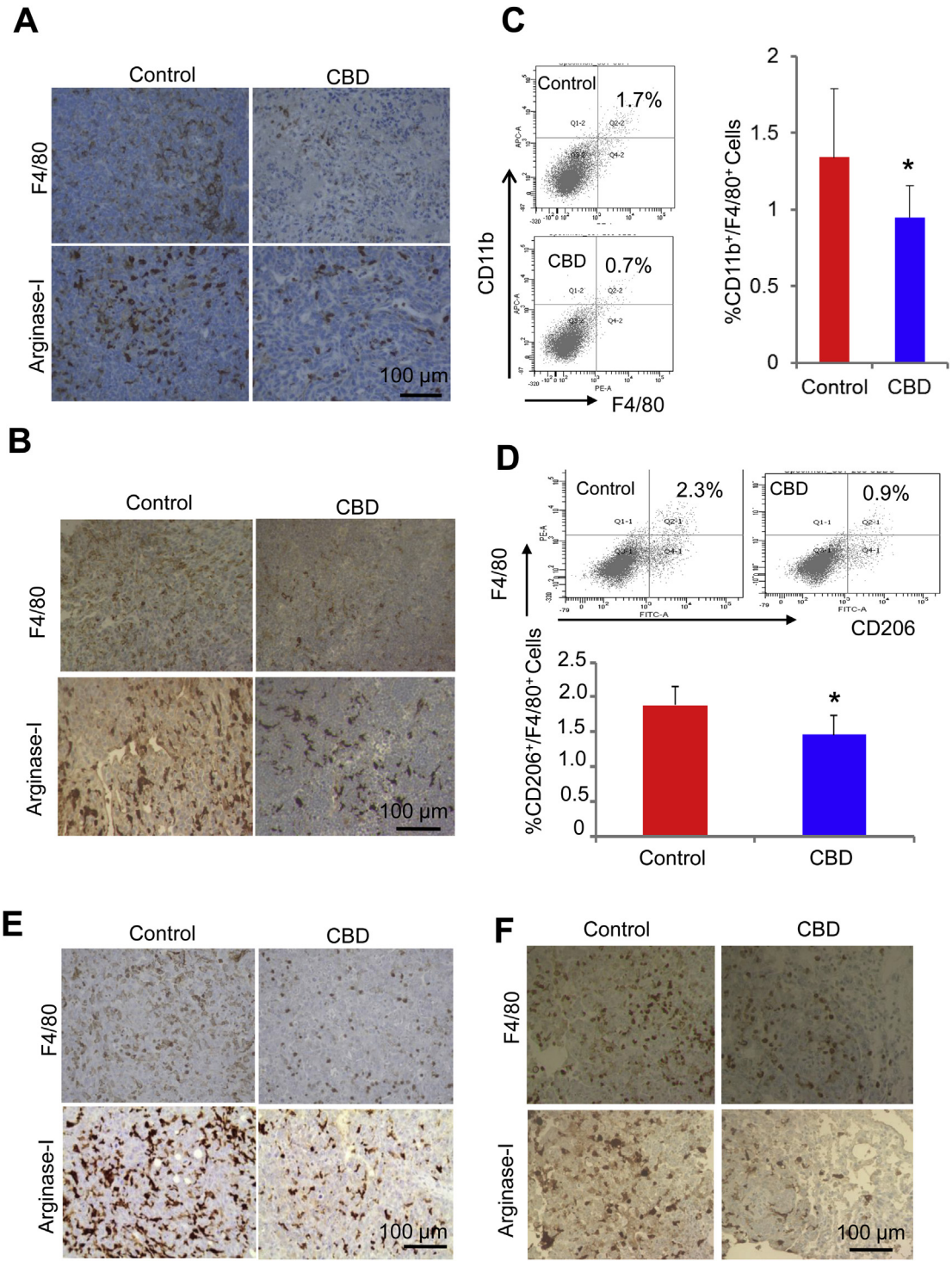

Figure 6 – CBD inhibits macrophage recruitment to the breast tumor microenvironment. Representative IHC photomicrographs of control and CBD-treated tumors of 4T1.2 (A) and MVT-1 B) mouse models using F4/80 and Arginase-I antibodies. Percentages of $CD11b^+/F4/80^+$ (C) and $CD206^+/F4/80^+$ (D) cells in control and CBD-treated tumors in MVT-1 mouse model. Representative IHC photomicrographs of lungs of control and CBD-treated groups of 4T1.2 (E) and MVT-1 (F) mouse models using F4/80 and Arginase-I antibodies.

and activate invasion and metastasis of cancer cells (Jacob et al., 2013; Visse et al., 2003). Our results show that CBD inhibits actin stress fibers and focal adhesion formation. Furthermore, we showed that CBD downregulates MMP2 and MMP9 secretion in TNBC cell lines and tumor lysates. These findings provide insights into the ability of CBD to inhibit breast cancer cell migration and invasion and decreased number of metastatic lung nodules in CBD-treated mice.

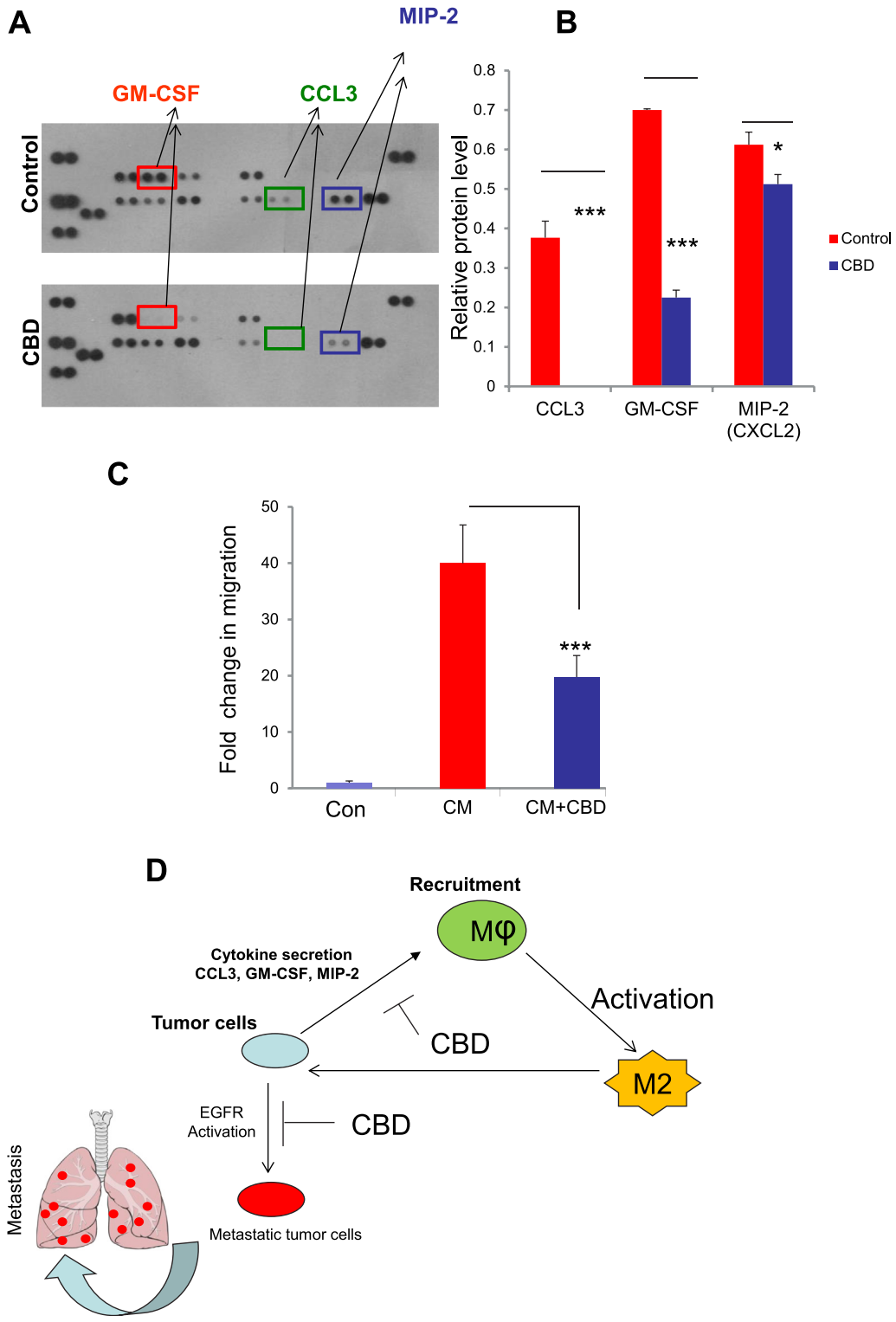

Figure 7 – CBD inhibits macrophage recruitment through modulation of breast cancer cell cytokine profile. (A) 4T1.2 cells were treated with vehicle or CBD for 48 h and conditioned media were used for cytokine profiling. (B) Quantification of cytokine array data using Image-J software for the affected cytokines. Data represent protein levels relative to loading controls. (C) Relative RAW 264.7 cells migration towards SFM (Con) or the conditioned media of vehicle-treated (CM) and CBD-treated (CM-CBD) 4T1.2 tumor cells (D) A diagram shows a putative anti-proliferative and anti-metastatic mechanism of action of CBD.

Our *in vivo* experiments confirmed the inhibitory activity of CBD on tumor growth and metastasis at 10 mg/kg doses. This finding is in accordance to McAllister et al., who showed that CBD reduced breast cancer metastasis through down-regulation of ID-1 and up-regulation of ID-2 (McAllister et al., 2011). The lack of significant tumor weight reduction in their study might be due to use of lower doses of CBD (5 mg/kg) and possibly due to variable sensitivity of different breast

cancer cell lines to CBD. In our study, the inhibitory effects of CBD on breast cancer xenografts resulted in decrease of lung metastases, decrease in tumor cell proliferation (as measured by Ki67 expression) and tumor vascularity (as measured by cell vessel density, CD31 expression). Moreover, CBD-treated tumors showed less activation of EGFR, AKT and ERK proteins, which is in keeping with our *in vitro* findings that CBD inhibits the EGF/EGFR signaling transduction pathway and its downstream targets.

The tumor microenvironment is well known to modulate tumor progression and the response to cancer treatment (Place et al., 2011). Tumor associated macrophages (TAMs) have been shown to secrete EGF and facilitate angiogenesis, degradation of ECM, and invasion of tumor cells (Schedin et al., 2007; Condeelis and Pollard, 2006; Wyckoff et al., 2004). M2 macrophages have been known to inhibit adaptive immune response and enhance stromal invasion through production of EGF and VEGF. Furthermore, M2 macrophages secrete IL-17 and various MMPs, which improve cancer cell stromal invasion (Zhu et al., 2008). Our study shows that CBD inhibits total macrophage and M2 macrophage populations within both primary tumors and secondary tumor metastatic sites. This might be due to inhibition of the recruitment of macrophages to the tumor site which has been confirmed by showing significantly less number of migrated mouse macrophage cell line (RAW 264.7) towards cancer cell conditioned media that was treated with CBD. This finding suggests that CBD may change the cytokine profile secreted from the cancer cells leading to decreased recruitment of macrophages to the tumor sites. We confirmed that by analyzing the affected cytokines secreted from 4T1.2 cell line after CBD treatment. We found that CBD inhibits CCL3 and GM-CSF secretion. This finding confirms our hypothesis as CCL3 is an important chemokine that is involved in macrophage recruitment and enhancement of lung metastasis (Wu et al., 2008). Furthermore, high GM-CSF is associated with $CCL18^{+}$ macrophages which induces cancer metastasis and reduces patient survival (Su et al., 2014).

Taken together, these finding show that CBD can suppress the activation of EGF/EGFR signaling transduction pathway and its downstream targets AKT, ERK and NF-kB. It is likely that CBD, through acting on its receptors, changes the cytokine secretion of cancer cells (less GM-CSF, CCL3). In turn, decreased recruitment of macrophages to the tumor microenvironment suppresses angiogenesis and inhibits the invasive potential of tumor cells. Eventually tumor cells display decreased proliferative and metastatic ability (Figure 7-D).

5. Conclusion

Overall, these results suggest CBD as a potent anti-tumor drug with anti-proliferative, anti-migratory, and anti-invasive properties. These results also suggest a cross-talk between EGFR and one of the receptors that CBD acts on. Furthermore, CBD has a tumor microenvironment modulating property which suggests an important role of CBD receptors on changing the cytokine profile within the tumor microenvironment. This study advocates the use of CBD in breast cancer patients especially those with highly aggressive and metastatic cancer cells including TNBC patients, and those who have resistance to conventional EGFR therapy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.molonc.2014.12.010.

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